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Purification and characterization of a dual function 3-dehydroquinate dehydratase from *Amycolatopsis methanolica*

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Studies on hydroaromatic metabolism in the actinomycete *Amycolatopsis methanolica* revealed that the organism grows rapidly on quinate (but not on shikimate) as sole carbon- and energy source. Quinate is initially converted into the shikimate pathway intermediate 3-dehydroquinate by an inducible NAD^+-dependent quinate/shikimate dehydrogenase. 3-Dehydroquinate dehydratase subsequently converts 3-dehydroquinate into 3-dehydroshikimate, which is used partly for the biosynthesis of aromatic amino acids, and is partly catabolized via protocatechuate and the β-ketoadipate pathway. Enzyme studies and analysis of mutants clearly showed that the single 3-dehydroquinate dehydratase present in *A. methanolica* has a dual function, the first example of a 3-dehydroquinate dehydratase enzyme involved in both the catabolism of quinate and the biosynthesis of aromatic amino acids. This enzyme was purified over 1700-fold to homogeneity. Its further characterization indicated that it is a Type II 3-dehydroquinate dehydratase, a thermostable enzyme with a large oligomeric structure (native M_r 135×10^3) and a subunit M_r of 12×10^3. Characterization of aromatic amino acid auxotrophic mutants of *A. methanolica* suggested that genes encoding 3-dehydroquinate synthase and 3-dehydroquinate dehydratase are genetically linked but their transcription results in the synthesis of two separate proteins.

Introduction

Quinate is utilized as a growth substrate by fungi as well as by bacteria and is metabolized via 3-dehydroquinate and 3-dehydroshikimate. These compounds are also intermediates in the shikimate pathway for the biosynthesis of aromatic amino acids (Fig. 1).

In organisms able to grow on both quinate and shikimate the initial step in the metabolism of both substrates generally appears to be catalysed by one and the same enzyme (Giles *et al.*, 1985). In Gram-negative bacteria this enzyme was characterized as a pyrroloquinoline quinone (PQQ)-dependent quinate dehydrogenase (van Kleef & Duine, 1988) while Gram-positive bacteria (including nocardioform actinomycetes) and fungi employ an NAD^+-dependent quinate dehydrogenase (Bruce & Cain, 1990; Cain, 1972*b*, 1981). *Neurospora crassa* and several species of rhodococci are able to grow on quinate but not on shikimate, probably because in these organisms shikimate is not an inducer of the quinate/shikimate dehydrogenase (Cain, 1981; Chaleff, 1974).

The dehydration of 3-dehydroquinate to 3-dehydroshikimate is catalysed by two different types of dehydroquinate dehydratase, distinguishable on the basis of enzymological and biophysical properties (Kleanthous *et al.*, 1992). The Type I enzymes characterized thus far have a solely biosynthetic function in the shikimate pathway and are expressed constitutively. A representative example is the *Escherichia coli* enzyme, a dimeric (subunit M_r 29×10^3) and heat-labile protein (Chaudhuri *et al.*, 1986). In *Aspergillus nidulans* and *N. crassa* the Type I enzyme is part of a pentafunctional complex of shikimate pathway enzymes (*AROM* complex) (Lumsden & Coggins, 1977; Charles *et al.*, 1985). During growth on quinate these organisms employ an additional Type II enzyme, an inducible, dodecameric (subunit M_r 18×10^3) and heat-stable protein (Giles *et al.*, 1967, 1985; Cain, 1972*a*; Lamb *et al.*, 1990; Kleanthous *et al.*, 1992). Recently, it has become clear that Type II enzymes may also function as biosynthetic enzymes in the shikimate pathway of *Streptomyces coelicolor* (White *et al.*, 1990) and *Mycobacterium tuberculosis* (Garbe *et al.*, 1991), organisms unable to grow on quinate. Many nocardioform actinomycetes are able to grow on quinate

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Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; PQQ, pyrroloquinoline quinone.

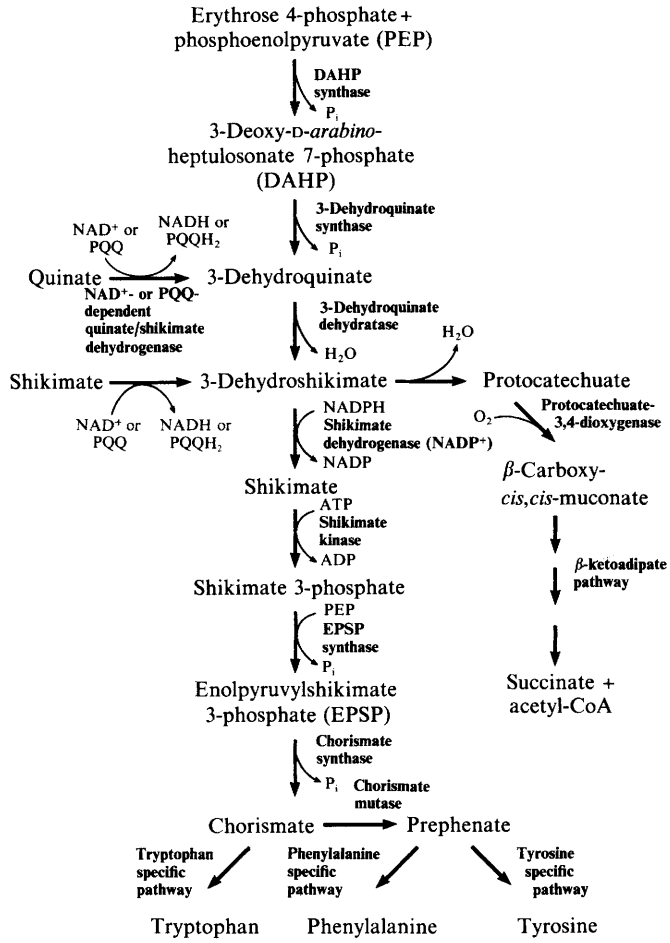

Fig. 1. Schematic representation of the biosynthesis of aromatic amino acids via the shikimate pathway and the catabolism of quinate and shikimate.

and studies on cell-free extracts indicated that these organisms may employ a single Type II dehydroquinate dehydratase with a dual function in quinate catabolism and in the shikimate pathway (Cain, 1981).

We are currently engaged in an analysis of the enzymology and regulation of the shikimate pathway enzymes in the methanol-utilizing actinomycete *Amycolatopsis methanolica* (previously known as *Nocardia* sp. 239) (de Boer *et al.*, 1990). Recently, Grund *et al.* (1990) reported that various *Amycolatopsis* species are able to use quinate as a carbon- and energy source. Quinate turned out to be an excellent growth substrate for *A. methanolica* as well. In this paper we report an analysis of quinate catabolism and the purification and characterization of a Type II 3-dehydroquinate dehydratase with a dual function in *A. methanolica*. Quinate catabolism can seriously interfere with attempts to overproduce aromatic amino acids via the shikimate pathway (Lamb *et al.*, 1991, 1992). The results allow a clear identification of targets for mutational inactivation of quinate catabolism.

Methods

Micro-organisms and cultivation. Amycolatopsis methanolica NCIB 11946, its maintenance and the procedures for cultivation in batch cultures have been described previously (de Boer *et al.*, 1989). Glucose was heat-sterilized, quinate and shikimate were filter-sterilized, and all three were added to final concentrations of 10 mM. A plasmid-cured derivative, strain WV2 (J. W. Vrijbloed, unpublished) was used in all experiments.

Respiration studies with whole cells. Cells from the mid-exponential phase of growth were harvested by centrifugation at 6000 *g* for 10 min at room temperature, washed five times with mineral medium and resuspended in mineral medium. Respiration rates were determined with a polarographic oxygen probe at 37 °C. The reaction was started by addition of substrate to a final concentration of 10 mM. Protocatechuate was added to a final concentration of 2·5 mM. The specific respiration rate was expressed as nmol O_2 min^{-1} (mg protein)$^{-1}$.

Mutant isolation. Auxotrophic mutants blocked in the shikimate pathway were isolated using the diepoxyoctane treatment as previously described (de Boer *et al.*, 1988). In the screening procedure glucose mineral medium was supplemented with aromatic amino acids, each to a final concentration of 1 mg l^{-1}. Pinpoint colonies were selected for further characterization.

Shikimate-utilizing spontaneous mutants of strain WV2 were

isolated in liquid mineral medium cultures with shikimate as sole carbon- and energy source. Growth was observed after incubation for approximately a week on a rotary shaker at 37 °C. After plating on shikimate agar, single mutants were isolated and characterized.

Extract preparation and enzyme assays. Cells from the mid-exponential phase of growth were harvested by centrifugation at 6000 *g* for 10 min at 4 °C, washed five times in 50 mM-Tris/HCl, pH 7·5, and resuspended in the same buffer. Cells were disrupted in a French pressure cell at 1000 MPa. Unbroken cells and debris were removed by centrifugation at 40000 *g* for 30 min at 4 °C. The supernatant, containing 2·5–5·0 mg protein ml^{-1}, was used for enzyme assays at 37 °C.

Quinate dehydrogenase and shikimate dehydrogenase (NAD^+-dependent; EC 1.1.1.24) were assayed according to Bruce & Cain (1990).

3-Dehydroquinate synthase (EC 4.6.1.3) activity was assayed using the *E. coli* 3-dehydroquinate dehydratase and $NADP^+$-dependent shikimate dehydrogenase as coupling enzymes and monitoring the oxidation of NADPH at 340 nm. The assay mixture contained 50 mM-Tris/HCl, pH 8·5, 1 mM-$CoCl_2$, 0·25 mM-NAD^+, 0·05 mM-NADPH, 0·2 U 3-dehydroquinate dehydratase (Chaudhuri *et al.*, 1987*b*), 0·2 U $NADP^+$-dependent shikimate dehydrogenase (Chaudhuri *et al.*, 1987*a*), 0·02 mM-3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) and extract in a total volume of 1·0 ml.

3-Dehydroquinate dehydratase (EC 4.2.1.10) activity was assayed according to White *et al.* (1990) at pH 8·5.

Shikimate:$NADP^+$ 3-oxidoreductase ($NADP^+$-dependent shikimate dehydrogenase) (EC 1.1.1.25) was assayed in the reverse direction by monitoring the oxidation of NADPH at 340 nm. The assay mixture contained 50 mM-Tris/HCl pH 8·5, 2 mM-shikimate, 0·15 mM-NADPH and extract in a total volume of 1·0 ml.

3-Dehydroshikimate dehydratase (EC 4.2.1.?) was assayed by following the formation of protocatechuate at 290 nm (molar extinction coefficient $3{\cdot}89 \times 10^3$ M cm^{-1}) (Bruce & Cain, 1990; Strøman *et al.*, 1978) or the disappearance of 3-dehydroshikimate at 234 nm (molar extinction coefficient 12×10^3 M cm^{-1}).

Protocatechuate-3,4-dioxygenase (EC 1.13.11.3) was assayed by monitoring the consumption of oxygen with a polarographic oxygen probe. The assay mixture contained 50 mM-Tris/HCl, pH 7·5, 2·5 mM-protocatechuate and extract in a total volume of 3·0 ml.

NAD^+-dependent shikimate- and quinate dehydrogenase were visualized after gel electrophoresis under non-denaturating conditions by quinate- or shikimate-dependent tetrazolium activity staining. Crude extracts of quinate-grown cells were loaded on a 7% (w/v) polyacrylamide gel in 100 mM-glycine/Tris, pH 9·0. After electrophoresis (100 V, constant current, 4 °C), the gels were stained in staining buffer, containing 60 mg NAD^+, 1 mg phenazine methosulphate (PMS), 15 mg 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 60 mg shikimate or quinate in a total volume of 30 ml. The gel was incubated in the assay mixture at 37 °C in the dark.

Partial purification of dehydroquinate synthase, dehydroquinate dehydratase, $NADP^+$-dependent shikimate dehydrogenase, NAD^+-dependent shikimate dehydrogenase and NAD^+-dependent quinate dehydrogenase. Crude extracts from either glucose-grown cells or quinate-grown cells of *A. methanolica* strain WV2 were applied on a Mono Q HR 5/5 anion-exchange column. Bound protein was eluted with an increasing linear gradient of 0–0·5 M-NaCl in 50 mM-Tris/HCl, pH 7·5. Fractions of 0·5 ml were collected.

Purification of 3-dehydroquinate dehydratase

Enzyme purification was done at room temperature unless stated otherwise. Centrifugation steps were done at 4 °C. All chromatographic steps were carried out with a System Prep 10 liquid-chromatography system (Pharmacia LKB).

Step 1. Glucose-grown cells were harvested in the stationary phase (82 g wet weight). Cell-free extract was prepared as described above. DNAase and 1 mM-$MgCl_2$ were added to the cell-free extract, which was then incubated for 10 min.

Step 2. Ammonium sulphate precipitation. Solid $(NH_4)_2SO_4$ was added slowly to the cell-free extract (194 ml; step 1) to a final concentration of 40 % saturation (43·84 g). The mixture was stirred for 30 min at 4 °C and centrifuged for 30 min at 40000 *g*. The supernatant (208 ml) was decanted and slowly adjusted to 60% saturation $(NH_4)_2SO_4$ (24·96 g). The mixture was stirred for 30 min at 4 °C and centrifuged for 30 min at 40000 *g*. The supernatant was discarded and the pellet was dissolved in 45 ml 50 mM-Tris/HCl, pH 7·5 (buffer A), and dialysed overnight against 5 l buffer A at 4 °C.

Step 3. The dialysed fraction from step 2 was incubated for 10 min at 70 °C. Precipitated protein was removed by centrifugation for 30 min at 40000 *g*. The pellet was washed in buffer A and centrifuged for another 30 min at 40000 *g* at 4 °C. The two supernatants were combined (93 ml).

Step 4. Two portions of the heat-treated material (46·5 ml) were applied onto a column of Q-Sepharose fast-flow (HR 10/10, bed volume 8 ml) equilibrated in buffer A. Bound protein was eluted with a two-step increasing linear gradient (0–0·25 M-NaCl in 30 ml and 0·25–0·5 M-NaCl in 100 ml) at a flow rate of 1·0 ml min^{-1}; fractions of 3·0 ml were collected. Peak fractions from both separate steps were pooled and dialysed against 5 l buffer A. This sample was concentrated on the same Q-Sepharose fast-flow column and eluted with an increasing linear gradient from 0–0·5 M-NaCl at a flow rate of 2·0 ml min^{-1} in 240 ml. Fractions of 3·0 ml were collected and peak fractions were pooled (49 ml) and dialysed against 5 l buffer A.

Step 5. Two portions of protein from step 4 were applied onto a Mono Q HR 5/5 anion-exchange column equilibrated in 50 mM-Tris/HCl, pH 8·5 (buffer B). Bound protein was eluted with an increasing linear gradient from 0–0·5 M-K_2HPO_4 in 60 ml at 1·0 ml min^{-1}. Fractions of 1·0 ml were collected and peak fractions from these steps were pooled (19·0 ml).

Step 6. Protein from step 5 was adjusted to 1·0 M-$(NH_4)_2SO_4$ and applied onto a phenyl-Superose HR 5/5 hydrophobic-interaction column equilibrated in buffer B with 1·0 M-$(NH_4)_2SO_4$. Bound protein was eluted with a decreasing linear gradient from 1·0–0 M-$(NH_4)_2SO_4$ in 45 ml at 0·5 ml min^{-1}. Fractions of 1·0 ml were collected and peak fractions were pooled (6·5 ml).

Step 7. Protein from step 6 was incubated for 10 min at 70 °C. Precipitated protein was pelleted in an Eppendorf centrifuge for 10 min.

Step 8. Protein from step 7 was concentrated to 200 µl in an Amicon ultrafiltration device (cut-off: M_r 10000) and applied onto a Superose 12 HR 10/30 column equilibrated in buffer B. Protein was eluted at a flow rate of 0·5 ml min^{-1}. Fractions of 0·25 ml were collected and peak fractions were pooled (3·8 ml).

Step 9. Protein from step 8 was applied onto a Mono Q HR 5/5 anion-exchange column equilibrated in buffer B. Bound protein was eluted with an increasing linear gradient from 0–0·5 M-NaCl at a flow rate of 0·5 ml min^{-1} in 30 ml. Fractions of 0·5 ml were collected. Fractions were pooled and glycerol was added to a final concentration of 20% (v/v) before storing at −20 °C.

SDS-PAGE. This was done according to Laemmli & Favre (1973) with the following marker proteins: phosphorylase A (M_r 94×10^3), human transferrin (80×10^3), albumin (68×10^3), catalase (58×10^3), fumarase (50×10^3), citrate synthase (46×10^3), carbonic anhydrase (31×10^3) and ribonuclease A (16×10^3). Gels were stained with Coomassie brilliant blue R250.

Estimation of molecular mass. The M_r of the native enzyme was estimated using a Superose 12 HR 10/30 column with thyroglobulin (M_r 670×10^3), gamma globulin (158×10^3), ovalbumin (44×10^3),

myoglobin (17×10^3) and cobalamin ($1{\cdot}35 \times 10^3$) as gel-filtration standards (Bio-Rad).

Automatic amino acid sequence determination. In two separate experiments the purified protein was transferred by electroblotting to Problot membranes (Matsudaira, 1987) and Amido-Black-stained dehydroquinate dehydratase bands were sequenced by Dr M. Cusack (Department of Pure and Applied Geology, University of Glasgow, UK) and Mr B. Dunbar (Department of Molecular and Cell Biology, University of Aberdeen, UK) on Applied Biosystems model 470A/120A automated gas-phase sequencers equipped with on-line HPLC for detection of the phenylthiohydantoin amino acid derivatives.

Kinetic studies with 3-dehydroquinate dehydratase. Kinetic parameters were determined at 37 °C and pH 8·5 or pH 7·5. The kinetic parameters were determined with the Enzfitter program (Elsevier software) using a direct fit.

Protein determination. Protein in crude extracts was determined with the Bio-Rad protein determination kit, using bovine serum albumin as a standard (Bradford, 1976). Protein in whole cells was determined after sonication of the cells for 10 min in the presence of 2 M-NaOH.

Inactivation with $NaBH_4$. Purified 3-dehydroquinate dehydratase was incubated for 40 min at room temperature with 50 mM-$NaBH_4$ in the presence or absence of 3-dehydroquinate. The incubation mixture contained: 1·0 U 3-dehydroquinate dehydratase, 1·0 mM-3-dehydroquinate, 50 mM-Tris/HCl, pH 8·5, and 50 mM-$NaBH_4$ (from a 1·0 M stock solution in 50 mM-NaOH) in a total volume of 1·0 ml. After 40 min the reaction mixture was diluted to 2·5 ml and immediately transferred onto a Pharmacia PD 10 desalting column; elution was with 3·5 ml 50 mM-Tris/HCl, pH 8·5. In a control experiment 50 mM-$NaBH_4$ was omitted and only 2·5 mM-NaOH was added.

Inhibition studies with aromatic amino acids on purified 3-dehydroquinate dehydratase. Aromatic amino acids were added separately or in combinations to a final concentration of 0·1 mM to the reaction mixture. 3-Dehydroquinate was added to a final concentration of 250 μM.

Chemicals. An *A. methanolica* strain WV2 mutant lacking 3-dehydroquinate synthase was found to accumulate DAHP intracellularly. Following preparation of cell-free extracts protein was precipitated with 5% (w/v) perchloric acid and the neutralized supernatant used directly as a source of DAHP for enzyme assays. The concentration of DAHP in the solution was determined enzymically, using dehydroquinate synthase, dehydroquinate dehydratase and $NADP^+$-dependent shikimate dehydrogenase, purified from *E. coli* (Coggins *et al.*, 1987). 3-Dehydroquinate was synthesized from D-quinate (Grewe & Haendler, 1968). 3-Dehydroshikimate was prepared enzymically from 3-dehydroquinate (Coggins *et al.*, 1987). Shikimate, protocatechuate and L-phenylalanine were purchased from Sigma. Quinate was obtained from Janssen Chimica, Belgium. NAD^+, $NADP^+$, L-tyrosine and L-tryptophan were obtained from Boehringer. $NaBH_4$ was obtained from Merck. Other chemicals were obtained from local industries.

Results and Discussion

Quinate and shikimate metabolism

The doubling time of *A. methanolica* strain WV2 in mineral medium with quinate as sole source of carbon and energy was 3·0 h. Quinate-grown cells were able to oxidize quinate, shikimate, 3-dehydroquinate, 3-dehydroshikimate and protocatechuate (Table 1). Growth on shikimate alone was not observed; also, addition of 10 mM-shikimate to cells growing on quinate did not result in increased final optical densities. Transfer of glucose-grown cells into a medium with quinate resulted in induction of quinate and shikimate dehydrogenase (both NAD^+-dependent) as well as protocatechuate dioxygenase activities while the $NADP^+$-dependent shikimate dehydrogenase appeared to be constitutive (Table 2). Mono Q anion-exchange chromatography failed to resolve the NAD^+-dependent quinate- and shikimate dehydrogenases although they were separated from the $NADP^+$-dependent shikimate dehydrogenase (Fig. 2). Addition of shikimate or quinate to NAD^+-dependent quinate dehydrogenase or shikimate dehydrogenase assay systems, respectively, did not result in enhanced activities. Non-denaturating PAGE of extracts, followed by tetrazolium-dependent staining for NAD^+-dependent quinate- and shikimate dehydrogenase activities invariably resulted in appearance of a single, identical band with both quinate and shikimate. Taken together, the above data suggest that a single NAD^+-dependent quinate/shikimate dehydrogenase is present in *A. methanolica*, as has been described for *N. crassa* (Barea & Giles, 1978), *A. niger* (Cain, 1972*b*) and *Rhodococcus rhodochrous* (Bruce & Cain, 1990). The

Table 1. *Oxygen consumption rates of glucose- and quinate-grown cells of A. methanolica strain WV2*

Activities are expressed in nmol O_2 min^{-1} (mg protein)$^{-1}$.

Substrate	Cells grown on: Glucose	Quinate
Glucose	170	30
Quinate	20	420
3-Dehydroquinate	0	295
3-Dehydroshikimate	0	300
Shikimate	45	305
Protocatechuate	0	170

Table 2. *Enzyme activities in crude extracts of glucose- and quinate-grown cells of A. methanolica strain WV2*

Activities are expressed in nmol min^{-1} (mg protein)$^{-1}$. ND, Not detectable.

Enzyme	Cells grown on: Glucose	Quinate
Quinate dehydrogenase (NAD^+)	0	220
Shikimate dehydrogenase (NAD^+)	0	175
3-Dehydroquinate dehydratase	205	210
3-Dehydroshikimate dehydratase	ND	ND
Protocatechuate dioxygenase	0	170
Shikimate dehydrogenase ($NADP^+$)	25	30

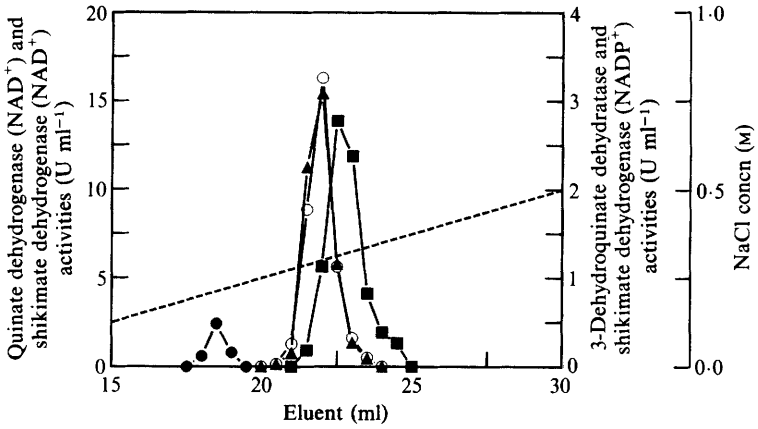

Fig. 2. Mono Q anion-exchange chromatography of crude extract of quinate-grown cells of *A. methanolica* strain WV2. Activity profiles of NAD^+-dependent quinate dehydrogenase (▲), NAD^+-dependent shikimate dehydrogenase (○), 3-dehydroquinate dehydratase (■) and $NADP^+$-dependent shikimate dehydrogenase (●). ---, NaCl gradient.

Table 3. *Enzyme activities in crude extracts of three shikimate-utilizing mutants of A. methanolica strain WV2 grown on glucose or shikimate*

Activities are expressed in nmol min^{-1} (mg protein)$^{-1}$.

Strain	Carbon source (10 mM)	Shikimate dehydrogenase (NAD^+)	Quinate dehydrogenase (NAD^+)	Protocatechuate-3,4-dioxygenase	Shikimate dehydrogenase ($NADP^+$)
S3	Glucose	255	270	0	30
S5	Glucose	315	165	0	30
S6	Glucose	220	170	0	30
S3	Shikimate	225	235	110	30
S5	Shikimate	280	370	75	40
S6	Shikimate	435	390	150	35

Table 4. *Purification of 3-dehydroquinate dehydratase from glucose-grown cells of A. methanolica strain WV2*

	Protein (mg)	Specific activity [U (mg protein)$^{-1}$]	Total activity (U)	Purification factor (-fold)	Yield (%)
1. Crude extract	1426	0·70	997	1	100
2. 40–60% $(NH_4)_2SO_4$	1037	0·67	696	1	70
3. 70 °C	237·8	2·45	583	4	59
4. Q-Sepharose	26·2	17·1	449	25	45
5. Mono Q	8·08	39·2	317	56	32
6. Phenyl-Superose	0·728	359	262	514	26
7. 70 °C	0·578	437	253	626	25
8. Superose 12	0·416	533	222	763	22
9. Mono Q	0·155	1200	186	1717	19

enzyme is clearly different from the constitutive shikimate dehydrogenase, which is exclusively $NADP^+$-dependent.

Although all enzymes necessary for the breakdown of shikimate are present in quinate-grown cells, no growth of *A. methanolica* on shikimate was observed, apparently because shikimate is unable to induce their synthesis. Shikimate is clearly able to enter the cells because low shikimate-dependent oxygen consumption rates are observed in glucose-grown cells (Table 1).

Shikimate-utilizing mutants of *A. methanolica* strain WV2 were readily isolated following incubation of the organism in shikimate mineral medium. The doubling times of mutant strains S3, S5 and S6 on shikimate were estimated as 2·1, 3·5 and 2·0 h, respectively. Their further characterization revealed that glucose-grown cells of these three mutants already possessed high NAD^+-dependent quinate/shikimate dehydrogenase activities, indicating that synthesis of the enzyme had become constitutive (Table 3). Protocatechuate dioxygenase was present in quinate- (Table 2) or shikimate-grown cells only (Table 3), suggesting that 3-dehydroshikimate or protocatechuate itself is the inducer of this enzyme. Addition of shikimate (10 mM) to quinate (10 mM)

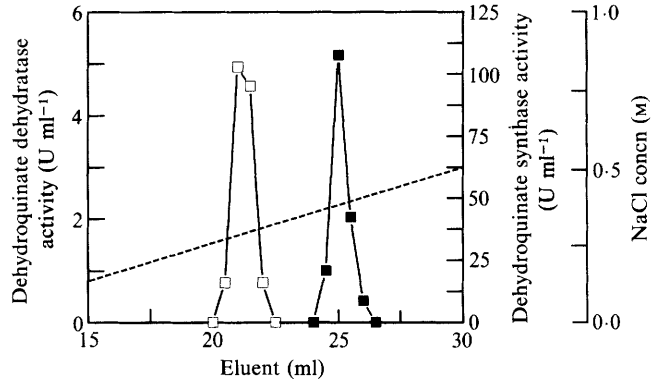

Fig. 3. Mono Q anion-exchange chromatography of crude extract (16·9 mg protein) of glucose-grown cells of *A. methanolica* strain WV2. Specific activities of dehydroquinate synthase and dehydroquinate dehydratase were 8 mU (mg protein)$^{-1}$ and 203 mU (mg protein)$^{-1}$ respectively. Activity profiles of 3-dehydroquinate synthase (□) and 3-dehydroquinate dehydratase (■) are shown. ---, NaCl gradient. A yield of over 80% was obtained.

mineral medium now resulted in an approximate doubling of the final cell densities of the mutant strains.

3-Dehydroquinate dehydratase was present at similar activity levels in glucose- and quinate-grown cells of strain WV2 (Table 2). In both cases the enzyme was heat stable and no loss of activity occurred during incubation for at least 10 min at 70 °C. Interestingly, when glucose- or quinate-grown cells reached the stationary phase, 3-dehydroquinate dehydratase became derepressed, resulting in a 3-fold increased specific activity (Table 4). Mono Q anion-exchange chromatography of crude extracts of glucose- or quinate-grown cells revealed only single activity peaks for 3-dehydroquinate dehydratase (Figs 2 and 3). These observations suggest that only a single 3-dehydroquinate dehydratase is present in *A. methanolica*.

3-Dehydroshikimate, the branch-point intermediate (Fig. 1), is converted to either protocatechuate (by 3-dehydroshikimate dehydratase) or to shikimate (by NADP$^+$-dependent shikimate dehydrogenase). Although quinate-grown cells clearly oxidized 3-dehydroshikimate (Table 1), 3-dehydroshikimate dehydratase activity was not detected in crude extracts (Table 2) using the assays previously described (Strøman *et al.*, 1978; Bruce & Cain, 1990). Attempts to measure 3-dehydroshikimate-dependent oxygen consumption in crude extracts, due to the formation of protocatechuate by 3-dehydroshikimate dehydratase activity combined with the conversion of protocatechuate by protocatechuate dioxygenase, also failed.

There are indications that in *Acinetobacter calcoaceticus* 3-dehydroquinate dehydratase and NADP$^+$-dependent shikimate dehydrogenase are present in a loose enzyme complex; this may allow channelling of 3-dehydroshikimate to NADP$^+$-dependent shikimate dehydrogenase rather than to dehydroshikimate dehydratase (Tresguerres *et al.*, 1972). In plants, these two enzyme activities actually constitute a bifunctional protein with probably the same channelling function (Polley, 1978; Fiedler & Schultz, 1985; Mousdale *et al.*, 1987). Mono Q anion-exchange chromatography of crude extracts of *A. methanolica* resulted in clear separation of 3-dehydroquinate dehydratase and NADP$^+$-dependent shikimate dehydrogenase activities (Fig. 2), although a loose association of these proteins cannot be excluded.

Isolation of mutants defective in 3-dehydroquinate dehydratase

Amongst a series of several phenylalanine, tyrosine plus tryptophan requiring auxotrophic mutants of *A. methanolica* strain WV2, three isolates (mutant strains GH7, GH12 and GH29) completely lacked both 3-dehydroquinate dehydratase and 3-dehydroquinate synthase activities. Various other isolates (mutant strains GH1, GH3, GH22) were blocked in 3-dehydroquinate synthase only. Mutants with a single block in dehydroquinate dehydratase, however, were not obtained. The data thus indicate that the 3-dehydroquinate synthase and 3-dehydroquinate dehydratase genes in *A. methanolica* are genetically linked; however, their transcription clearly results in the synthesis of two separate proteins (Fig. 3). Such linkage has been reported for the *M. tuberculosis* genes (Garbe *et al.*, 1991). Mutant strains GH7, GH12 and GH29 were unable to grow on quinate as sole carbon- and energy source, even in media supplemented with all three aromatic amino acids. Mutant strains GH1, GH3 and GH22 grew normally in quinate mineral medium without requiring supplementation by aromatic amino acids; in glucose medium quinate could replace the three aromatic amino acids as growth supplements. These observations provide further evidence that one and the same 3-dehydroquinate dehydratase enzyme is involved in quinate catabolism and the biosynthesis of aromatic amino acids in *A. methanolica*.

Purification of 3-dehydroquinate dehydratase

3-Dehydroquinate dehydratase was purified from glucose-grown cells of *A. methanolica* strain WV2 that had

entered the stationary phase (Table 4). As mentioned above, the specific activity of the enzyme increased considerably in this growth phase, reaching a level of 700 mU (mg protein)$^{-1}$. The heat stability of this 3-dehydroquinate dehydratase is quite pronounced (no loss of activity during incubation at 70 °C for 10 min) and simplified its purification (steps 3 and 7). 3-Dehydroquinate dehydratase activity eluted at 300 mM-NaCl from a Q-Sepharose column. High-resolution anion-exchange and hydrophobic interaction chromatography, followed by gel filtration and a second anion-exchange chromatography step subsequently were necessary to obtain a pure protein. The use of phosphate ions instead of chloride ions in step 5 resulted in a better resolution of protein peaks in this particular case. 3-Dehydroquinate dehydratase was purified 1717-fold with a total yield of 19%, yielding a homogeneous preparation as judged by SDS-PAGE (Table 4).

Table 5. *Yield of phenylthiohydantoin amino acid at each stage of the automated protein sequencing*

Cycle no.	Residue	Yield (pmol)
1.	Met	50
2.	Lys	23
3.	Val	40
4.	Phe	49
5.	Val	46
6.	Leu	45
7.	Asn	38
8.	Gly	35
9.	Pro	27
10.	Asn	25
11.	Leu	24
12.	Gly	22
13.	Arg	13
14.	Leu	23
15.	Gly	22
16.	Lys	6
17.	Arg	12
18.	Glu	16
19.	Pro	13
20.	Ala	13

Properties of 3-dehydroquinate dehydratase

The M_r of the native enzyme was estimated as $135 \times 10^3 \pm 12 \times 10^3$. SDS-PAGE analysis revealed the presence of a single subunit with an M_r of 12×10^3, indicating a dodecameric quaternary structure for the enzyme. The optimum conditions for 3-dehydroquinate dehydratase activity were found to be 76 °C and pH 9·0. The K_m values of the enzyme for 3-dehydroquinate were 121 ± 5 μM at pH 7·5 and 266 ± 12 μM at pH 8·5. The estimated V_{max} values were 690 U (mg protein)$^{-1}$ at pH 7·5 and 1607 U (mg protein)$^{-1}$ at pH 8·5. The enzyme was not inhibited by 0·1 mM-L-tryptophan, -L-tyrosine or -L-phenylalanine, nor by combinations of these three amino acids.

Type I 3-dehydroquinate dehydratases are irreversibly inactivated during incubation with $NaBH_4$ and 3-dehydroquinate because the imine intermediate involved in catalysis is reduced to a stable secondary amine (Chaudhuri *et al.*, 1991; Kleanthous *et al.*, 1992). Type II enzymes, including the biosynthetic dehydroquinate dehydratase of *S. coelicolor*, are not inhibited by this treatment since imine formation does not appear to be involved in the reaction mechanism (Kleanthous *et al.*, 1992). The *A. methanolica* enzyme turned out to be insensitive to $NaBH_4$, in the presence or absence of 3-dehydroquinate.

Table 6. *Properties and function(s) of 3-dehydroquinate dehydratases from various organisms*

Enzyme	Organism	K_m (μM)	Subunit M_r ($\times 10^{-3}$)	Structure	Function	Reference
Type I	*Escherichia coli*	18	29	Dimer	Biosynthetic	Chaudhuri *et al.* (1986)
	Neurospora crassa	5	165*	Dimer	Biosynthetic	Coggins *et al.* (1987)
	Aspergillus nidulans	–	171*	Dimer	Biosynthetic	Charles *et al.* (1986)
	Plants	27	59†	Monomer	Biosynthetic	Mousdale *et al.* (1987)
Type II	*Neurospora crassa*	70	18·5	Dodecamer	Catabolic	Hautala *et al.* (1975)
	Aspergillus nidulans	150	16·5	Dodecamer	Catabolic	Kleanthous *et al.* (1992)
	Streptomyces coelicolor	650	16	Dodecamer	Biosynthetic	White *et al.* (1990)
	Mycobacterium tuberculosis	–	14‡	Dodecamer‡	Biosynthetic	Garbe *et al.* (1991)
	Amycolatopsis methanolica	121§	12	Dodecamer	Biosynthetic/ catabolic	

* Part of the *AROM* complex.
† Part of the 3-dehydroquinate dehydratase/NADP$^+$-dependent shikimate dehydrogenase bifunctional enzyme.
‡ Personal communication from A. R. Hawkins.
§ Determined at pH 7·5 and 37 °C.

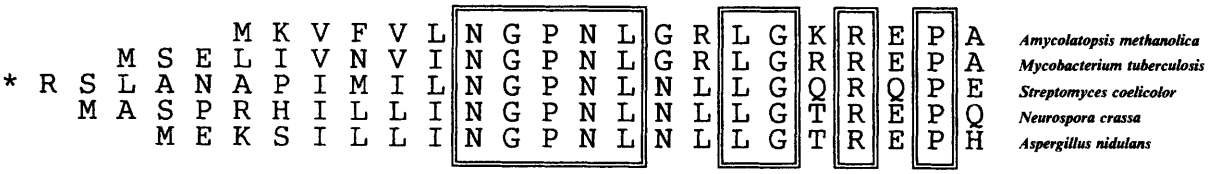

Fig. 4. Alignment of the N-terminal sequences of Type II 3-dehydroquinate dehydratases purified from various organisms. *Not identified.

N-Terminal amino acid sequence analysis initially allowed unambiguous identification of the first eight residues only; in a second experiment (results shown in Table 5) this sequence was confirmed and extended to 20 residues. No homology was found with the Type I biosynthetic 3-dehydroquinate dehydratase from *E. coli* (Duncan *et al.*, 1986). Instead, alignment of the first 20 N-terminal amino acids of the 3-dehydroquinate dehydratase from *A. methanolica* with previously published sequences from Type II enzymes revealed 45% overall identity with the catabolic enzymes from *N. crassa* (Hawkins *et al.*, 1982) and *A. nidulans* (Da Silva *et al.*, 1986) and the biosynthetic enzymes from *S. coelicolor* (White *et al.*, 1990) and *M. tuberculosis* (Garbe *et al.*, 1991) (Fig. 4). Highest homology was observed with the *M. tuberculosis* enzyme (75% identity). Compared to the Type I *E. coli* enzyme, all five Type II enzymes have small subunits, are thermostable and have high K_m values for 3-dehydroquinate (Table 6). Amongst these Type II enzymes the *A. methanolica* 3-dehydroquinate dehydratase is somewhat unusual in its relatively low subunit M_r, its intermediate K_m value, and its dual function in both quinate catabolism and the biosynthetic shikimate pathway.

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